CYCLIC CO_2 RELEASE IN DIAPAUSING AGAPEMA PUPAE

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One of the most engaging recent problems in insect respiration is posed by the following observations: (1) Certain insects release CO_2 in brief periods of very rapid output ("bursts") alternating with long periods of very slow output (Punt, 1944, 1950). (2) In such insects O_2 uptake is continuous and constant throughout periods much exceeding the length of the burst cycle (Schneiderman and Williams, 1953, 1955). (3) Though the R.Q. may be 0.1 or less during the interburst period (O_2 taken up 10 or more times as fast as CO_2 is released), the R.Q. computed over an entire cycle is similar to that in many insects with continuous CO_2 release (0.78, Schneiderman and Williams).

The continuity of O_2 uptake shows that the cycle cannot be due to any type of rhythmic change in over-all metabolic rate. The conventional over-all R.Q. indicates that the CO_2 released in the burst should be regarded not as gas suddenly generated at that time but as the normal respiratory CO_2, *produced* at a steady rate throughout the cycle but somehow retarded in *escape* except during the brief burst period. Punt had stated that there were no body movements corresponding to the bursts, and had ascribed the rhythmic release of CO_2 to a corresponding rhythm of spiracular activity. He apparently assumed that entry of O_2 would be reduced by the spiracles whenever escape of CO_2 was retarded, which is not true. Whether the CO_2 retention could be accomplished by cyclic spiracular activity *without* interference with O_2 uptake will appear from the following consideration: Assuming that gas transfer is by diffusion, the quantity passing through the spiracles in unit time is given by Fick's Law: $Q = [D(C^o - C^i)A]/L$, where Q is the rate of gas transfer, D is the diffusivity of the gas in question, C^o and C^i, the concentrations outside and inside the spiracle, A, the area of the aperture of the spiracular valve, and L, the length of the valve lumen in the direction of diffusion. Because O_2 and CO_2 travel the same path, pore area and length can be neglected and relative transfer rates will be determined only by diffusivities and concentration gradients:

$$\frac{Q_{O_2}}{Q_{CO_2}} = \frac{k_{O_2}\,(C^o{}_{O_2} - C^i{}_{O_2})}{k_{CO_2}\,(C^i{}_{CO_2} - C^o{}_{CO_2})}.$$

Since the diffusivities of O_2 and CO_2 differ only in the ratio of 5:4 it is clear that the spiracles could not, by themselves, bring about the observed 10-fold or more disparity in transfer rates of CO_2 and O_2 during interburst. Accordingly it seems necessary to restudy the CO_2 cycle with special reference to spiracular activity, the triggering of the burst, and the interrelations of the variables of the cycle (burst volume, cycle length, interburst release rate).

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Materials and Methods

Diapausing 600–1400 mg. pupae of the saturniid moth *Agapema galbina* were used in most of the work, confirmatory tests being made on 3–5 g. pupae of the saturniid *Rothschildia orizaba*. (CO_2 release was also measured in 9 diapausing pupae of the phalaeniid moth *Admetovis oxymoris* over an average of 20 hours per individual without any discontinuity being observed.) *Agapema,* which pupates (in southern Texas) in December and normally emerges in September and October, was studied from January through May in two successive years, and *Rothschildia* which pupates (in southern California) in September and emerges in May and June, was studied in March and April. Since the two batches of *Agapema* differed somewhat in a number of respects, some of the data are given separately by year. Parasitism by larvae of certain flies, prevalent in saturniid pupae, was shown not to affect the results (Buck and Keister, 1955).

Gas exchange was measured at 5-minute intervals throughout periods of up to 72 hours by Warburg's direct method, O_2 uptake in the presence of 10% KOH, and CO_2 release (by difference) in the presence of 5.5% H_2SO_4, the concentration of acid having the same vapor pressure of water as the alkali. The *Agapema* pupae were rested on the insets of 15-ml. flasks, the liquid being in the flask bottoms, whereas *Rothschildia, Samia* and *Platysamia* were tested in 100-ml. flasks with the volume reduced to about 30 ml. with paraffin, and with the liquid in small watchglasses. Manometers were shaken at about 65 cycles per minute for improved temperature control. Because of the necessity of making continuous records of CO_2 output over many hours and the impossibility of measuring O_2 uptake simultaneously by Warburg manometry, O_2 uptake and CO_2 release in a given individual were usually measured on alternate days. For pupae in full diapause, this practice seems justified by the extremely slow rates of change in weight and metabolic rate, and the rate of CO_2 release during the bursts is so much higher than that of O_2 uptake that no appreciable error is introduced in that part of the cycle. However, a proportionally much greater uncertainty is involved in the computed interburst release rates because the CO_2 is appearing at a rate so much lower than the O_2 uptake rate assumed to apply at that time. Each animal was examined for body movement and heartbeat after each test, to make sure it was alive, since notable bacterial gas exchange can be recorded from dead specimens.

In sealing experiments a resin adhesive (Rebel No. 502, Southern Adhesives Corp.) was used, one-half hour in air being allowed for drying. Unless otherwise stated, respiration measurements were made at 25° C. "Q_{O_2}" and "Q_{CO_2}" indicate rates of gas exchange per unit live weight.

Results

1. *General pattern of CO_2 release*

Data from more than 1900 bursts in 124 pupae in which CO_2 release was measured for an average of 109 hours per individual are summarized in Tables I–III, and in Figure 1. Some idea of the degree of variation between *Agapema* pupae in the same batch, and between batches, can be obtained from Table I, which gives the frequency distribution of mean cycle length in the 124 individuals in runs averaging about 8 hours. *Rothschildia* pupae were similarly variable, ranging

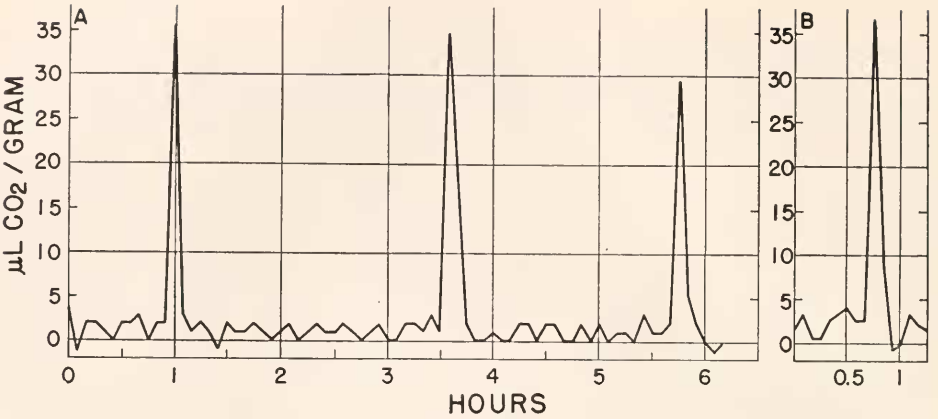

FIGURE 1. Typical records of CO_2 release rate in *Agapema* (A) and *Rothschildia* (B), showing CO_2 bursts; 25°.

from individuals giving small bursts every half hour or so to those giving large bursts at longer than 8-hour intervals. The particular 1953 figures used in Table I cover a period of only a few weeks; later, after various experiments had been run, there had been marked increases in burst frequency in some individuals, several giving bursts as often as every hour, whereas in a few pupae there was a decrease in mean frequency. The 1954 figures involve a longer time span, but in spite of this there was a much larger proportion of long-period pupae than in 1953.

TABLE I

Number of pupae of given mean CO_2 release cycle length at 25°

Batch	Total no. pupae	Mean cumulated hours of obs. per pupa*	Cycle lengths in hours						No bursts
			1–2	2–3	3–4	4–5	5–6	6+	
1953	35	46	0	3	13			17	2
1954	89	47	3	5	3	11	5	60	2

* Ranging from 8–120 hours per individual, but mostly from 20–60 (*e.g.*, the standard deviation for the 1954 pupae was 22.2 hours).

Cycle variation within a given pupa during a period of continuous measurement is indicated by the data for burst volume and cycle length in Table II.

For rough comparison with Schneiderman and Williams' data on the pupa of the Cecropia moth, *Platysamia,* Table III gives the variables of the CO_2 burst cycle in a number of multiple-burst pupae for which corresponding O_2 uptake records were available. From this it appears that *Agapema* pupae differ from those of *Platysamia* in having bursts which are briefer and represent only 16–36 times the volume of CO_2 released continuously in an equal (10 minute) period (rather than up to more than 200-fold) and in releasing only 45–65% of their CO_2 production per cycle as bursts (rather than up to 95%). R.Q.s ranging from 0.4 to 5.0 are

obtainable, depending on the period in the cycle chosen for calculation, the average figures for a complete cycle being 0.65 and 0.81 for the small samples treated in Table III. In most of our discussions a value of 0.73 will be assumed.

2. *O_2 uptake in diapausing Agapema pupae*

Concerning the important question of normal O_2 uptake rate, we concluded after careful study that Schneiderman and Williams are correct in reporting a steady and continuous O_2 uptake during periods when CO_2 bursts are occurring. Small but statistically significant perturbations were in fact seen in some of our O_2 uptake records, but can be regarded as artifacts due to inability of the alkali

TABLE II

Average CO_2 burst volume and cycle length per individual Agapema pupa at 25°

	No. pupae	No. bursts	Mean burst vol. μL/g.	% volume variation*	No. cycles	Mean cycle length (hours)	% length variation*
1953	9 (22 series)	79	28.6	16.8±2.2	58	3.26	16.0±2.1
1954	29 (42 series)	118	26.1	19.4±2.3	87	4.96	21.2±3.7

* Per cent variation from mean was computed for each series of 2–5 consecutive cycles in one individual. All these were then averaged.

to absorb instantaneously all the CO_2 from bursts occurring just before the time when the manometer was read.

In experiments with various O_2-N_2 mixtures we found Q_{O_2} constant throughout the range 1%–100% O_2. This is important in indicating the unlikelihood of the pupa being hypoxic at any stage in the cycle.

3. *Exclusion of non-spiracular gas-exchange*

In confirmation of Schneiderman and Williams' finding, both O_2 uptake and CO_2 release were practically zero in pupae with all spiracles sealed. This indicates that cutaneous respiration is negligible and that the continuous low-level CO_2 release of interburst must occur via the spiracles, as Punt thought.

Though the anus is not ordinarily functional in lepidopteran pupae we took the precaution of testing the effect of thoroughly sealing the anal end of the pupa.

TABLE III

CO_2 burst cycle variables in Agapema (25°)

(*IB = interburst*)

	No. pupae	Mean wt.	Q_{O_2} (μL/g./hr.)	Burst duration (min.)	Burst vol. (μL/g.)	Cycle length (hrs.)	IB rate (μL/g./hr.)	Total IB CO_2 per cycle (μL/g.)	Burst rate/ IB rate	Burst as % of Σco_2 per cycle
1953	13	.85 g.	30	10	33.3	3.6	11.9	42.8	17	44
1954	13	.81 g.	13	14	32.0	4.5	3.8	17.1	36	65

In 15 such pupae 23 bursts occurred in 10 hours, compared with 30 bursts in 14 hours on the preceding day, indicating that intestinal gas is not involved in the CO_2 bursts.

4. *Role of the spiracles*

Since Punt had attributed the cyclic changes in CO_2 release rate to spiracular activity, we investigated this point (*cf.* also Buck, Keister and Specht, 1953). The spiracles of *Agapema* are marked externally by hard, oval stigmata, each of which consists of a rigid thickened border, the peritreme, enclosing a flat immov-

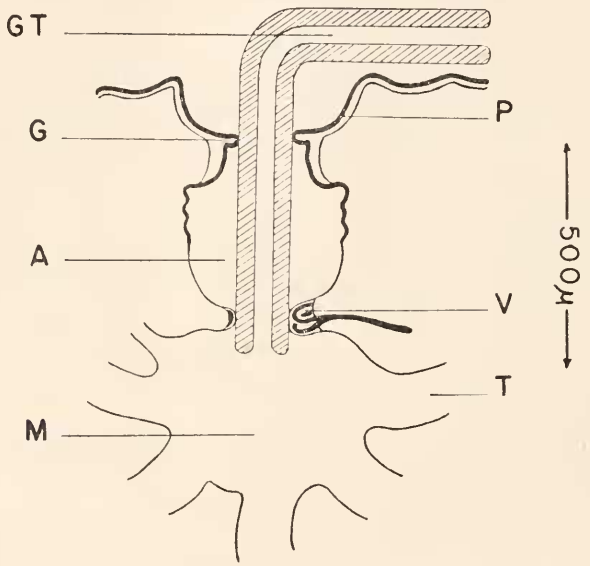

FIGURE 2. Diagram to show glass tube (GT) forced through grating (G) within the peritreme (P) so that it passes through the spiracular atrium (A) and the valve (V) into the tracheal manifold (M).

able plate pierced by a narrow slit with serrated edges. If the stigmal plate is chipped away it can be seen that about 500 μ in from the body surface of the pupa the large tubular atrium is flattened so that the passage is closed. Dissection shows that the flattening is brought about by the apposition of movable sclerotized bars surrounding the atrium in such a way as to make an effective valve (V, Fig. 2). Internal to the valve lies a roughly spherical "manifold" chamber (M) from which a dozen or more large tracheae branch out into the viscera.

Under the conditions necessary for observation (removal of peritreme; use of spotlight), the valve opens momentarily to an elliptical slit at intervals considerably shorter than the usual cycle length. It is not known whether this is normal behavior, and it has thus far been impossible to watch the activity of the valves in the living animal during actual respirometry. However, the effect of inactivating the spiracular valve was tested by inserting a capillary glass tube (Fig. 3) through

the stigma to a point beyond the valve (Figs. 2, 4, 5). The tube was about 175 μ in outside diameter, fire-polished to prevent injury to the respiratory intima, and provided with a right-angle bend to prevent its penetration deeper than about 600 μ. The lumen of the tubing was about 75 μ, but since the lips of the valve do not close tightly around the cannula a solid rod would probably serve as well. Theoretically

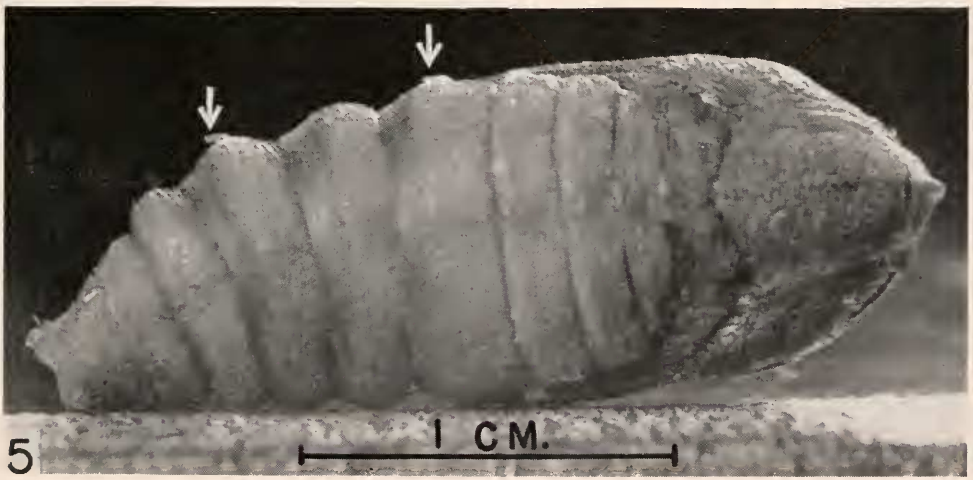

FIGURE 3. Fire-polished glass tubes used for spiracular intubation (on millimeter rule).
FIGURE 4. Tubes in place in 2d and 3d abdominal spiracles of the left side. × 20.
FIGURE 5. Dorsal view of *Agapema* pupa showing tubes in place in 3d and 5th abdominal spiracles of the left side.

a single spiracular tube would be sufficient to keep the tracheal gas in contact with the external environment, since all 14 spiracles are interconnected by large tracheae, but to make sure, 4–6 abdominal spiracles were intubated in each *Agapema* pupa, and 3 in *Rothschildia*.

The effect of spiracular intubation was very dramatic. In 16 *Agapema* pupae not a single burst was recorded in 7½ consecutive hours, whereas the same individuals had given 38 bursts during 8 hours on the previous day. Confirmatory

results were obtained in *Rothschildia*. When the tubes were removed the pupae still produced no bursts, which was disconcerting until it was discovered microscopically that the tubes had warped the valves so that they could no longer close. When, thereupon, only the spiracles which had been intubated were sealed, the capacity to release CO_2 discontinuously was completely restored, and the pupae remained alive and apparently normal for several months thereafter.

5. *Relation of spiracles to water balance*

Since the intubation experiments indicated that the spiracles are concerned with CO_2 retention during interburst, we thought it relevant to see whether the escape

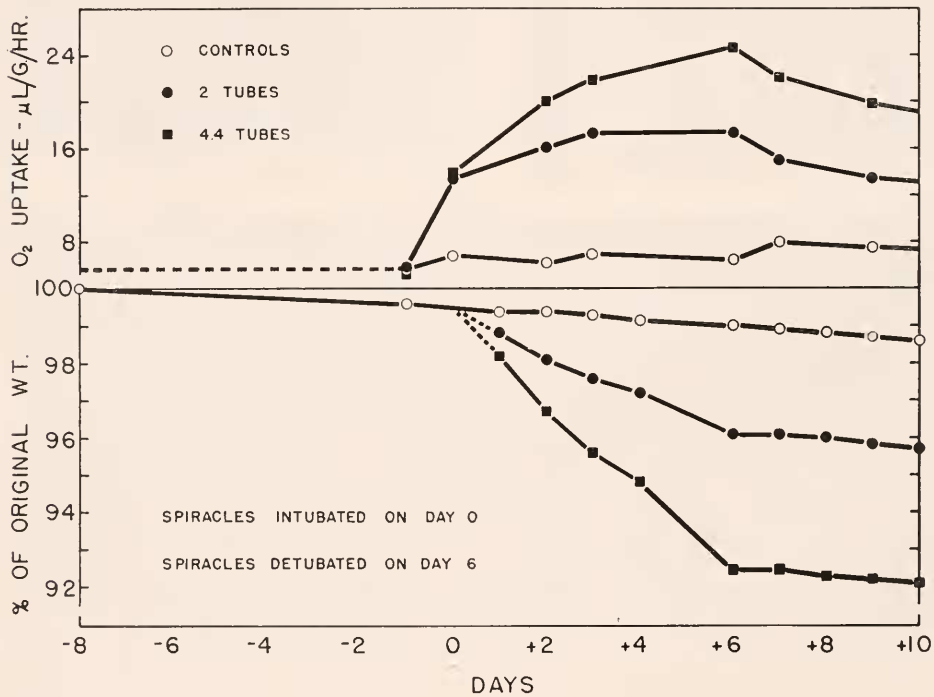

FIGURE 6. Rates of O_2 uptake (above) and weight loss (below) of pupae before, during, and after spiracular intubation, in comparison with controls; 25°.

of water vapor is likewise impeded. We have not yet been able to measure water loss separately in burst and interburst periods, but over-all rate of loss was readily ascertained from over-all weight loss corrected for loss of respired solids. Figure 6 gives the mean over-all weight loss and mean O_2 uptake rates of each of 3 groups of 5 pupae carefully selected to be practically identical in physical characteristics and burst history, and Table IV gives some average data on weight and water loss. As shown in Figure 6, normal weight loss in laboratory air is almost vanishingly small, amounting to less than 0.5% in 6 days. However, in the 6 days after

intubation the group with two spiracles per pupa intubated lost 3.5% of its initial weight, and the group with an average of 4.4 intubated spiracles per pupa lost 7%. When the tubes were removed and those spiracles sealed, the rates of weight loss reverted at once to the control level. The water loss under various conditions, corrected for fat burned (R.Q. taken as 0.73) is given in the last column of Table IV, and indicates that water loss increases roughly in proportion to the number of spiracles intubated.

The O_2 uptake rates given in Figure 6, the measurement of which required a total of 11% of the duration of the weight loss experiments, show that the intubation operation slowly stimulated respiration. This is in accord with Schneiderman and Williams' (1953) findings on the sustained effects of mechanical injury. It might be thought that the weight losses recorded were due to the stimulated respiration, rather than to water loss, but this is contraindicated by the linearity of weight loss in comparison with the decelerating increase in respiration, by the fact that the ratios of increase in O_2 uptake were not the same as the ratios of

TABLE IV

Weight and water loss in intubated pupae at 25°

Group (5 pupae each)	Initial wt./pupa (mg.)	Wt. loss per pupa per day (mg.)	Peak Q_{O_2}/ g. hr. (μL)	Total O_2 uptake per day per pupa* (μL)	Total O_2 uptake per day per pupa* (mg.)	H_2O from carbohydrate per pupa** (mg.)	H_2O from fat/ pupa** (mg.)	Carbohydrate loss per day per pupa*** (mg.)	Fat loss per day per pupa*** (mg.)	Water loss per per day pupa (from fat) (mg.)
Control	1027	1.0	5.64	139	0.2	0.11	.08	0.2	.07	.93
2 tubes	1078	6.0	17.26	447	.64	.36	.24	0.6	.22	5.8
4.4 tubes	1060	12.7	24.72	629	.9	.51	.34	.84	.31	12.4

* Computed from peak rate.

** *I.e.*, amount of metabolic water produced, assuming pure substrate respired.

*** *I.e.*, computed from over-all O_2 uptake, assuming pure substrate respired.

weight loss in the two experimental groups, and by the immediate return to the control rate of weight loss after detubation and sealing, as contrasted with the slow decline in Q_{O_2}. Furthermore, if possible substrate loss is computed as glucose, and the peak rate of uptake is taken as applying throughout the period of intubation, the loss of solids is only a trifling fraction of total weight loss (Column 9, Table IV). It can be concluded, therefore, that water vapor, as well as CO_2, is normally retained by the spiracles.

The very slow rate of rise and fall in Q_{O_2} gives assurance that rise in metabolic rate *per se* cannot be the cause of the sudden abrogation and restoration of ability to produce bursts in the intubation experiment.

6. *Working hypothesis of the CO_2 burst cycle*

Our demonstration that CO_2 retention is abolished when the spiracles are made inoperative does not prove that the spiracles normally control the burst cycle. Since direct observation during respirometry was not feasible we investigated burst volume, cycle length and interburst release rate under different conditions to see whether these variables are interrelated in ways which are compatible with spiracular

action.[1] This requires, however, a description of the cycle in relation to possible triggering of its various phases.

A priori the simplest interpretation of the burst cycle is perhaps that the low-level interburst CO_2 release represents leakage through closed or nearly closed spiracles and the burst represents the liberation, at the time of spiracular opening, of the CO_2 which accumulates in excess of that which can leak out. The spiracles would thus act as a sort of safety valve to prevent the internal CO_2 concentration from rising above a certain level. In terms of control, this interpretation of the burst cycle centers in the starting and in the stopping of the burst, events which seem most reasonably attributed to the opening and the closing of the spiracles. Spiracular closing would thus affect burst volume and burst duration, and spiracular opening would determine cycle length.

7. *Interrelations of cycle variables in individuals and populations*

If burst volume, cycle length and interburst release rate change under various conditions, their interrelations should bear not only on spiracular control but on the hypothesis that CO_2 is at least one of the factors controlling spiracular action, and on the rationale of discontinuous CO_2 release *per se*. Thus, for example, if a larger than average burst were released, due to delay in spiracular *closing* or to wider opening than usual during the burst, it might be expected that the succeeding interburst period would be longer than usual because a longer time would be required for the impounding CO_2 to build up the (abnormally low) internal concentration to the level required to trigger spiracular opening. On the other hand, if the burst were larger than average because of delayed spiracular *opening* (*i.e.*, the internal CO_2 concentration rose higher than usual before a burst), it would be expected that larger than usual bursts would be associated with longer than usual *pre*ceding interbursts.

Previous work has not given a clear answer on this point, possibly because of the very small number of records involved. Punt called attention to unusual burst size associated with unusually long *succeeding* interburst period, and Schneiderman and Williams believe that burst volume is correlated with length of *preceding* interburst period. In *Agapema* an analysis was made of over 300 cycles in 18 pupae, occurring in series of 2–9 consecutive cycles per individual, comparing cycles reckoned as a burst plus the interburst immediately preceding, and as the same burst plus the interburst immediately following. There was very considerable variation between and within individuals and no significant difference in mean magnitude was found in interburst length, total interburst volume or total cycle CO_2 release calculated on the two bases. This could mean either that spiracular opening is as variable as spiracular closing or that the two operations are correlated. At any rate, from the practical standpoint a cycle may apparently be defined as a burst plus either the preceding or succeeding interburst. In our computations of total cycle CO_2 output we have arbitrarily used the latter.

Even the most cursory examination shows that there is some regularity in burst volume and cycle length in successive CO_2 release cycles of a given pupa. However, the degree of variation is such, and the difficulty in measuring an adequate

[1] Burst duration is probably variable also, but the time resolution of the Warburg method is insufficient to detect such differences.

number of consecutive cycles in a single pupa is so great, that no statistically valid distinction between the *degree* of variability of the different cycle variables in an individual can be obtained directly. However, an analysis of variance of cycle variables of the 1954 pupae showed that the variance of burst volume is highly significantly less within individual pupae than between mean burst volume in different pupae. In contrast, cycle length, interburst CO_2 release rate and total interburst CO_2 volume all show about as much variation between different cycles of a single pupa as between pupae. This indicates that burst volume is much more constant than cycle length or interburst rate in successive cycles of an individual. Hence the lack of clear association of a burst with either preceding or succeeding interburst period may mean that the internal CO_2 level for spiracular closing varies

TABLE V

Correlation analysis of burst cycle variables in two samples of Agapema pupae (Q_{CO2} = av. rate of CO_2 release over whole cycle)

Pairing	1953 (29 cycles)			1954 (109 cycles)		
	r	*t*	*p*	*r*	*t*	*p*
1. Q_{O_2} vs. Q_{CO_2}	.91	11.4	<.001	.50	5.97	<.01
2. Q_{O_2} vs. IB rate	.60	3.90	<.001	.59	7.56	<.001
3. Q_{O_2} vs. B vol./g.	.20	1.06	>.20	−.20	2.11	>.02
4. Q_{O_2} vs. Σ_{CO_2}/cycle	−.20	1.06	>.20	−.20	2.11	>.02
5. Q_{O_2} vs. cycle length	−.64	4.32	<.001	−.54	6.64	<.01
6. Q_{CO_2} vs. IB rate	.79	6.7	<.001	.59	7.56	<.01
7. Q_{CO_2} vs. B vol./g.	.21	1.11	>.20	.15	1.57	>.05
8. Q_{CO_2} vs. Σ_{CO_2}/cycle	−.02	.104	>.90	−.04	.414	>.60
9. Q_{CO_2} vs. cycle length	−.62	4.10	<.001	−.52	6.3	<.01
10. IB rate vs. Σ_{CO_2}/cycle	.40	2.27	>.02	.21	2.22	>.02
11. IB rate vs. B vol./g.	.02	.104	>.90	−.14	1.46	>.10
12. IB rate vs. cycle length	−.16	.84	>.30	−.21	2.22	>.02
13. B vol./g. vs. Σ_{CO_2}/cycle	.30	1.6	>.10	.52	6.3	<.01
14. B vol./g. vs. cycle length	.16	.84	>.30	.28	3.02	<.01
15. CO_2/cycle vs. cycle length	.73	4.89	<.001	.63	8.4	<.01
16. B vol./g. vs ΣIB_{CO_2}	.15	.69	>.40	.12	1.25	>.10
17. wt. vs. Q_{O_2}	−.15	.70	>.50	−.22	1.61	>.10
18. wt. vs. absolute burst vol.	.53	2.86	<.01	.48	5.66	<.01

in parallel with that for opening so as to keep the total amount of CO_2 released in the burst fairly constant, even though the absolute triggering level (and cycle length) varies.

Turning now to data from populations, Table V summarizes a correlation analysis of burst cycle variables in two batches of *Agapema* pupae. Since only Q_{O_2}, burst volume and cycle length were measured directly, a conservative 1% level of significance seems desirable. With this criterion, there seems to be a significant inverse relation between metabolic rate and cycle length (pairings 5 and 9), the additional CO_2 apparently appearing mainly during interburst (pairings 2, 6) rather than in the bursts (pairings 3, 7). This is consistent with other indications of constancy or independence of burst volume (pairings 11, 13, 16) and with the analysis of variance in individuals. The relation between burst volume

and cycle length, an important one (pairing 14), seems to be clear enough in the 1954 population, namely that pupae with larger bursts have longer interbursts. The correlation is even more striking when means from pupae with cycles longer and shorter than 4 hours are compared ($p = < .001$). Nevertheless the lack of correlation in our 1953 population and the non-association between burst volume and Q_{O_2} (pairing 3), each of which is highly correlated with cycle length, are disturbing. Actually, the analysis summarized in Table V may weight the case against association since there are a number of entries for each short cycle pupa and only one for each long cycle one. If means of cycle variables for pupae with cycles shorter than 4 hours are compared with those for pupae with cycles longer than 4 hours, an inverse association between Q_{O_2} and burst volume significant by t test at the 2% level is obtained.

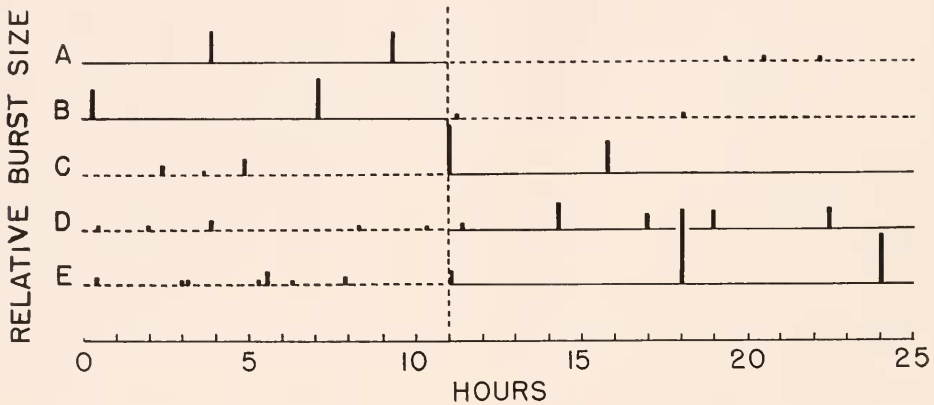

FIGURE 7. CO_2 burst pattern in pure O_2. Shift from air (solid horizontal lines) to pure O_2 (broken horizontal lines) made at 11 hours (broken vertical line). Pupae A and B had been in air with shaker, heaters and lights on for 12 hours (without recording) prior to time zero. Pupae C, D and E had been in O_2 with shaker, heater and lights on for 8 hours prior to time zero. Flasks were not flushed at time zero; 25°.

8. *Effects of temperature and p_{O_2} on the burst cycle*

Punt (1944, 1950) and Schneiderman and Williams (1955) have reported that both increased temperature and increased metabolic rate cause a progressive increase in burst frequency and interburst CO_2 release rate, and a decreased burst volume.

Schneiderman and Williams also reported that increasing the environmental O_2 concentration above that in air caused a progressive decrease in rate of interburst CO_2 release until, in 100% O_2, practically all the CO_2 was released in bursts. Decreasing the ambient O_2 had the opposite effect, bursts disappearing altogether at some concentration between 15% and 6%, *i.e.*, all the CO_2 produced was released continuously. Similar results were obtained with *Agapema,* except with pure O_2, in which bursts were suppressed almost to the vanishing point (Fig. 7). This effect, apparently different from that in *Platysamia,* was found consistently in two 36-hour experiments involving 30 pupae each, and alternated with respect to time of day and sequence of exposure to air and O_2.

No progressive effect of O_2 on either cycle length or burst volume is apparent in the records of Schneiderman and Williams nor in *Agapema* population means from different O_2 concentrations. The O_2 threshold for burst production, which in *Platysamia* lies between 15 and 6%, seems in *Agapema* to be about 10%. Thus, as shown in Table VI, the burst frequency in 13% O_2 is indistinguishable from that in air, and bursts are essentially absent in 7%. Of the 30 pupae tested in 10% O_2, 13 which had given two or more bursts in a comparable period in air on the preceding day gave no bursts at all, and two others ceased giving bursts in the course of the experiment.

The apparently rather abrupt cessation of burst production at about 10% ambient O_2 raises the questions whether there is a real break between continuous and discontinuous types of CO_2 release, and if so whether temperature and p_{O_2} differ qualitatively in their effects in this respect. Schneiderman and Williams' 1955 data indicate about 60% higher interburst release rate at 15% O_2 than in

TABLE VI

Frequency of burst production in different environmental O_2 concentrations. Same 30 pupae used in each test; duration of experiments, 18–23 hours; 30°

	13% O_2	Air, preceding day	10% O_2	Air, preceding day	7% O_2	Air, preceding day
No. pupae giving two or more bursts	23	21	5	25	0	27
No. pupae giving single bursts	6	8	10†	3	2‡	3
Total bursts in 18 hours	85	98	30*	135	2	147

† Six occurred within the first hour, hence were probably mechanically triggered (see p. 157).
‡ All occurred within the first hour.
* Almost all were very small.

air, but only three pupae are involved, and the range is above the critical region. In *Agapema* the variability was such that no statistically significant difference in any cycle variable could be established near the "threshold." Some evidence may be provided by the time course of experiments involving prolonged respirometry of multiple-burst pupae in the critical range. Thus Figure 8 shows a decrease in mean burst size of about 2% per hour, cycle length apparently being little affected (no sufficiently long continuous records of O_2 uptake were available for computing reliable interburst release rates). The progressive change in burst volume might be attributable either to the computed 2.2% fall in O_2 concentration or to the 1.6% rise in CO_2 in the flask atmosphere due to pupal respiration during 20 hours. Insofar as this type of evidence is relevant, therefore, there is no indication of a sharp change in mode of CO_2 release. However, it should be emphasized that the available temperature and O_2 data are too scanty and variable in both *Platysamia* and *Agapema* to give decisive quantitative information.

Further evidence of progressive changes in CO_2 retention with decreasing ambient p_{O_2} comes from experiments with pure N_2. Since burst production ceases gas exchange did indeed soon cease, but before this happened extra CO_2 was evolved at about 10% O_2, no burst would be expected during anoxia. In point of fact all

(Buck, Keister and Specht, 1953). Ordinarily this "purge" began when the flasks were flushed with N_2, thus being mainly lost in the equilibration period, but occasionally it was delayed as much as 15 minutes, permitting direct measure (Fig. 9). From such records it was found that purges differ from normal CO_2 bursts in being spread over 31 minutes (mean for 16 purges) and in involving over twice as much CO_2 as a normal burst of the same pupa, even when the flushing is performed immediately after a normal burst in air has occurred. A similar prolonged bleeding

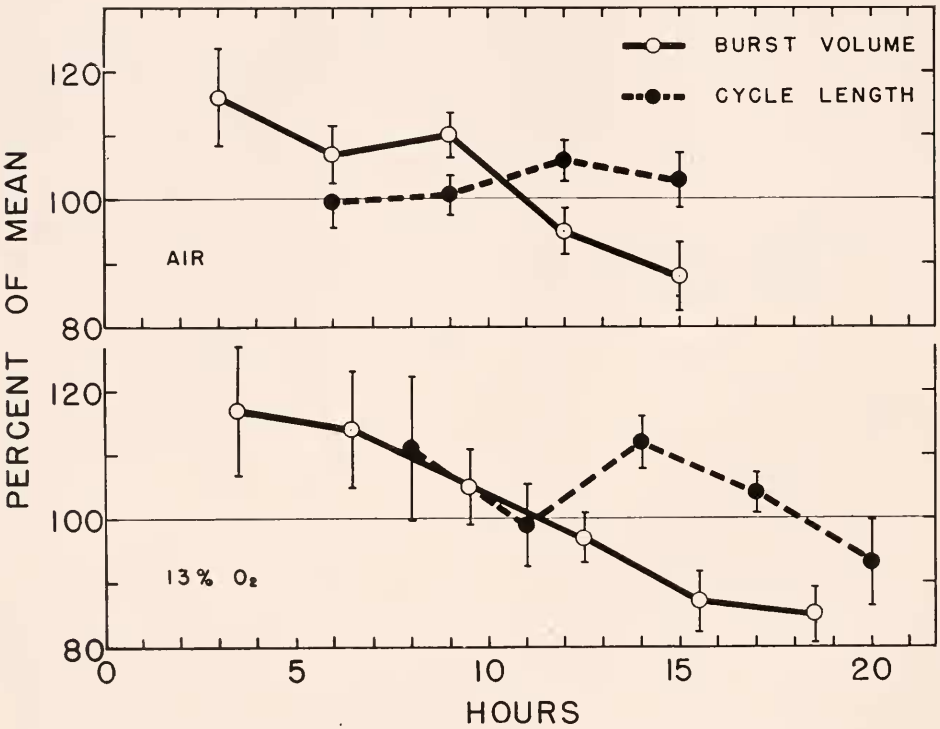

FIGURE 8. Cycle changes in long experiments; 30°. The burst plot was obtained by first plotting, for each pupa, each individual burst volume (as % of mean burst volume for the whole experiment for that pupa) at the time of its occurrence and connecting the points with straight lines. Then the intercepts at the selected 3½, 6½, 9½, etc. hour ordinates with each individual pupal plot were averaged and plotted. The cycle length plot was obtained similarly, plotting the individual cycle lengths (as % of pupal mean) at the time of occurrence of the burst ending the particular interburst period. All pupae gave three or more bursts, and there were 10 pupae in the 13% group and 22 in the control group.

off of CO_2 was seen after spiracular intubation. We interpret this to mean that there is normally present in the tissue and blood at all times a large reserve of CO_2 and CO_2 derivatives, and that in a burst the spiracles close again before all this stored CO_2 is released. In an anaerobic environment, or after intubation, on the other hand, the spiracles are forced to stay open, allowing additional CO_2 to diffuse out. Thus the first interburst period in air subsequent to a N_2-induced

purge, or sealing after detubation, is inordinately lengthened (Table VII), as might be expected if the triggering CO_2 concentration were not attained until both the CO_2 normally involved in the cycle and that in the depleted permanent reserve had been impounded. A similar interpretation might be applied to the casual statement of Punt (1944) that prolonged bursts involve longer than usual cycles.

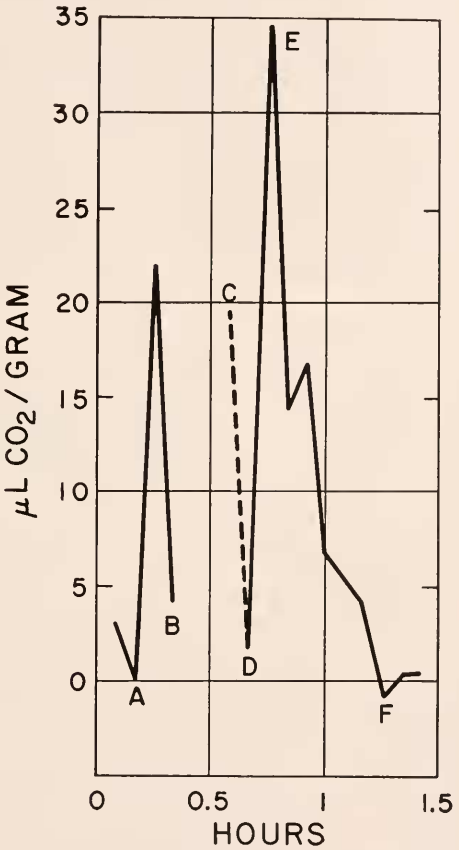

FIGURE 9. CO_2 purge produced by anoxia; 25°. A–B is the normal burst. At B the flask was flushed with pure N_2. C–D is the equilibration from flushing. At D the purge begins, reaching a greater than normal rate at E, and lasting about 3 times as long as the normal burst.

9. *Mechanical triggering of CO_2 release*

Though in normal burst production some internal factor related to respiration triggers the sudden release of CO_2, it was observed that in most experiments a significantly larger number of bursts occurred soon after setup than expected by chance. This is well shown in Figure 10, which gives the time distribution of 90 bursts in 55 records from multiple-burst 1953 pupae in related experiments. Furthermore, 11 of the pupae giving two or more bursts had interburst periods

TABLE VII

Effects of intubation and N_2 purging on cycle length at 25°

No. pupae	Mean cycle length on preceding day (hr.)	Mean length first cycle after detubation and sealing (hr.)	Mean length first cycle after purge (hr.)	% increase over preceding cycle length	Mean cycle length on day after detubation (hr.)
6*	2.7	4.8		78	2.0
6	4.0		6.2	55	

* Five additional pupae, for which no pre-intubation data were available, showed the sort of post-detubation increase in burst frequency indicated in the last column.

of the proper lengths to reach approximately zero time if extrapolated backwards from the time of occurrence of their ostensible first bursts. Assuming that the first observed bursts of these pupae were in fact their seconds, and that the true first bursts (indicated in solid black in Fig. 10) were lost during the setup and

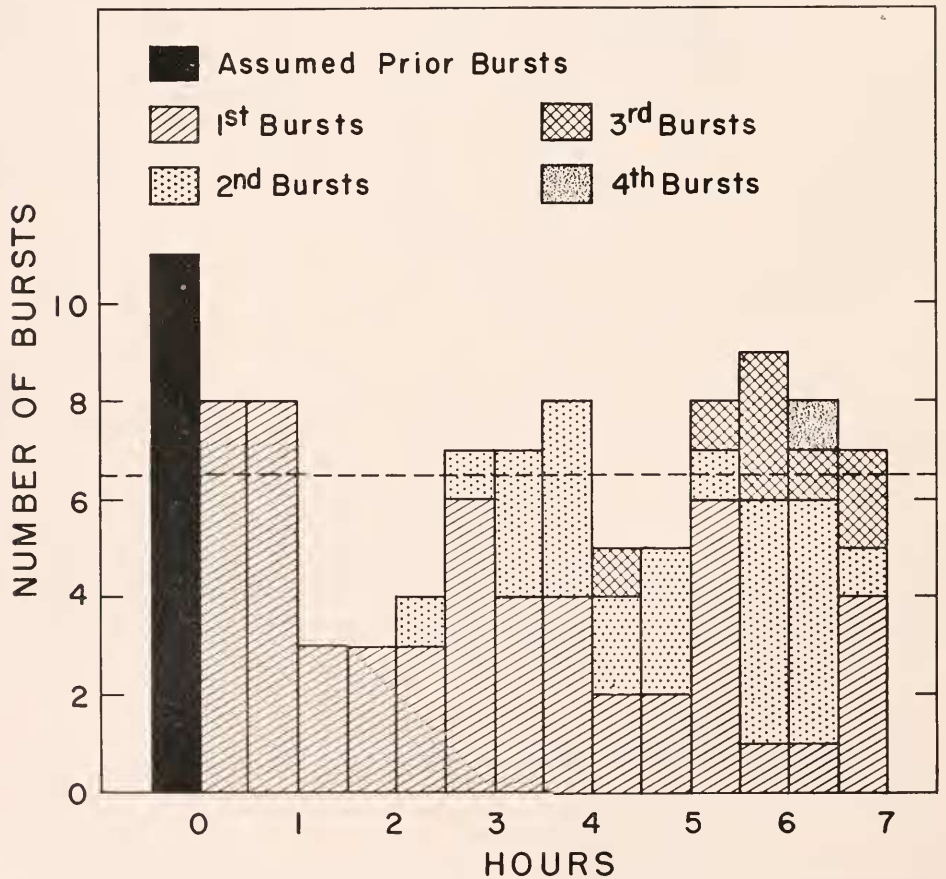

FIGURE 10. Temporal distribution and sequence of CO_2 bursts to show disproportionate number occurring within the first hour after the start of the experiment; 25°.

equilibration periods at the start of the experiment (the rhythms being "reset" from that point), the discrepancy between the number of bursts occurring during the first hour after setup and during the second becomes even more striking. The 1954 pupae gave a still more exaggerated response, the number of first bursts in the first hour ranging up to 75% in some experiments.

Burst frequency was apparently not correlated with time of day. There was some preponderance of first bursts in the first hour of an experiment in which pupae were left in the flasks all night with lights and shaker on and in which the only apparent environmental change at the start of the experiment was the closing of the manometer cocks. However, a much greater preponderance occurred after initial air-flushing of the flasks and a still greater excess after the usual initial handling of the pupae in weighing, insertion in flasks, etc. It is reasonable that handling might trigger the release of CO_2 because pupae often squirm at this time. However, it is strange that the bursts thus apparently induced can be delayed as much as an hour after the actual handling. At any rate the phenomenon needs to be kept in mind in analyses involving burst frequency and spacing.

Discussion

(a) *The control of CO_2 release and its possible relation to the spiracles*

The intubation experiment shows clearly that spiracular integrity is necessary for the occurrence of the CO_2 burst cycle. The discussion of cycle variations under experimental and metabolic change indicates that many of these responses are at least qualitatively consistent with spiracular involvement in control of CO_2 release. It is therefore reasonable to consider further the triggering of CO_2 release in possible relation to the spiracles.

In our discussion of the cycle as a hypothetical CO_2 regulator it was concluded that the triggering of the burst itself should depend on internal CO_2 concentration. This view is supported strongly by the lengthening of the first interburst period following a N_2 purge or intubation (Table VII), reminiscent of Wigglesworth's (1935) interpretation that the duration of the closed period of flea spiracles is determined in part by accumulation of CO_2. However, the observation that rate of CO_2 release during interburst is constant within the limitations of Warburg respirometry indicates that the change in internal p_{CO_2} during a cycle must be slight. The N_2 "purge" experiment, moreover, shows that much CO_2 remains in the body at the end of a normal burst. How solubility and tissue buffering of CO_2 could permit such relative constancy in the face of burst production will be considered in a separate communication.

The observed changes in CO_2 release rate with changing temperature and ambient p_{O_2} suggest that at least during interburst the aperture of the spiracular valve is influenced also by internal O_2 concentration. Thus circumstances which decrease the availability of O_2, such as a lowering of the environmental p_{O_2}, or increase the utilization of O_2, such as the increased metabolism due to higher temperature or breaking of diapause, would force the spiracles to open wider to provide more O_2. The enlarged opening would allow a more rapid escape of CO_2 which, in turn, would account for the observed increase in rate of interburst CO_2 release with decreased p_{O_2}. The N_2 purge results also fit this scheme, since this maximum

ambient O_2 deficiency, presumably inducing an extreme and also sustained opening of the spiracles, could be expected to permit the escape of most of the labile CO_2 in the body.

The possibility that the increased interburst CO_2 release rate with rising temperature is due to the increased rate of CO_2 production, rather than to increased port area, can be neglected if tissue buffers keep intratracheal p_{CO_2} fairly constant, as postulated.

In reference to stimulation of CO_2 release by O_2 deficiency, and also to the question of the progressive nature of the response, it is interesting to recall Schneiderman and Williams' (1955) report that in 6% O_2 ". . . the rate of carbon dioxide release increased markedly and sank to a lower level only after several hours." If the half-hour purge in N_2 (zero O_2) occurs through widely opened spiracles, and if spiracular valve area changes inversely with environmental p_{O_2}, the smaller opening associated with 6% O_2 might indeed be expected to "smear" out the purge over several hours (and, incidentally, to lead to a spurious R.Q. during this period).

Insofar as mode of triggering is concerned, there is ample precedent in other insects for spiracular opening with increased ambient p_{CO_2} (*e.g.,* Hazelhoff, 1926b, 1928), decreased ambient p_{O_2} (Hazelhoff, 1926b; Buck, 1948), or a combination of these gases (Wigglesworth, 1935; Case, 1954). In each instance it may reasonably be assumed that an ambient gas produces its effect via changes in the composition of intratracheal and tissue gas, and is thereby comparable to the triggering due to respiration in diapausing pupae. In view of the well recognized function of spiracles in reducing water loss in insects there is reason to suspect that water vapor also might influence valve activity. Hazelhoff (1926a), however, is reported by Punt (1944) to have found no difference in the opening of the spiracles in adult insects in moist and dry air, and Punt himself found no difference in the pattern of burst production in an adult *Triatoma* under such conditions.

The triggering of CO_2 release in diapausing pupae and its relation to spiracular activity thus emerges as a complex subject. Whatever the mechanism of CO_2 retention it is apparent that the normal cycle involves both an all-or-none type of response, during which occurs the abrupt, extreme and brief increase in CO_2 release rate which we call the burst, and long periods in which the rate is essentially static in spite of increasing retention. As we have seen, the data are insufficient to delineate the transition between the two states. There are, however, two reasons for thinking that the change is not as abrupt as the records suggest. The first is that the transition is partly a matter of the time-resolution of the respirometer. This is well shown by Punt's (1944) records at 22° and 36°, the discontinuity of which would certainly have been lost in Warburg manometry. The second is that if there is a reciprocal relation between ambient p_{O_2} and spiracular aperture it is not a linear one. Thus, from Fick's law, $A \propto \frac{1}{c^o - c^i}$ for constant Q_{O_2} where A = port area and c^o and c^i are the gas concentrations outside and inside. The relation is a rectangular hyperbola and means that at the high end of the p_{O_2} range large changes in O_2 concentration would require only relatively small changes in spiracular aperture, whereas at low O_2 concentrations small changes would induce large changes in spiracular area (causing a spurious break in CO_2 leak rate).

The few interpretable data on triggering could be taken to indicate that CO_2 controls the sudden opening of the spiracles at burst time, while O_2 regulates the valve position sustained during interburst and the rate of over-all release in the range of O_2 concentrations below the burst "threshold." However, in view of Wigglesworth's (1953) and Case's (1954) evidence for interaction of O_2 and CO_2 in stimulating spiracles it seems more likely that the level of tissue CO_2 may not be constant, as assumed in our simplified description of the cycle, but may vary with spiracular area (temperature, p_{O_2}, etc.), hence modifying both interburst release rate and triggering level. The analysis will be carried somewhat further on the theoretical side in another communication.

(b) *Rationale of the CO_2 release cycle*

Insofar as the rate of CO_2 release during interburst and the possible constancy of burst volume in the normal cycle are concerned, the results fit well with the concept of the burst cycle as alternating accumulation and escape of metabolic CO_2. The induced changes in cycle variables, however, are less easily integrated. Cycle length and burst volume do indeed change predictably with temperature, and each shows also an inverse relation with metabolic rate in populations, although this is not necessarily related to the effects of induced changes in individuals. However, the direction of change (decrease with rising temperature) does not seem easily reconcilable with either the anticipated constancy of burst volume or with the expectation that if interburst release rate increases it should take a longer, not a shorter, time to attain the triggering CO_2 concentration. The effects of changes in ambient p_{O_2} are even less well marked and consistent, particularly with concentrations above 21%. We are left, in fact, without a clear picture of either the triggering of the different phases of the cycle, or indeed a satisfactory rationale for the alternating retention and escape of CO_2.

We suggested previously (Buck, Keister and Specht, 1953) that the retention of CO_2 is not the prime objective in itself, but a consequence of provisions for minimizing transpiratory water loss. This idea is consistent with the well recognized role of the spiracles in water conservation in insects in general (*cf.* Hazelhoff, 1926b), with the particularly acute need for conservation in pupae (denied water intake for months or years), and with the demonstrated relation between spiracular integrity and water loss in *Agapema*. Furthermore it could furnish a reason for the sensitivity of the cycle to ambient p_{O_2} in that maximum water retention would require the diffusion port area to be the minimum compatible with adequate respiratory O_2 supply. From Table IV the daily control water loss per one gram pupa is about one mg. or 1360 μL, and the daily CO_2 loss (assuming an R.Q. of 0.73) is $5.64 \times 24 \times 0.73 = 100$ μL, or only $1/14$ of the water vapor loss. Alternatively, if we assume the water vapor loss of the pupae treated in Table III to be in the same proportion to body weight and Q_{O_2} as in those of Table IV, the water loss per cycle would be about 22 and 6 times the observed over-all CO_2 loss in the 1953 and 1954 pupae, respectively. However, the diffusivity of water is only 50% greater than that of CO_2. The validity of viewing the CO_2 release cycle as a consequence of water vapor retention must therefore await the determination of the respective concentration gradients.

Even though a one-mg. daily water loss (0.1% body weight) seems negligible, it would account for an unsupportable loss if maintained through a two-year dia-

pause. Presumably, however, the average rate of desiccation in nature is lower, even in this desert species of moth, because of long periods at temperatures less than 25° and because of maintenance of a higher-than-environmental humidity within the cocoon.

(c) *Mechanism of CO_2 retention*

Evidence has been presented that the spiracles are intimately involved at all stages of the CO_2 release cycle. In controlling the burst *per se* their role presents little difficulty, assuming that tracheal p_{CO_2} is higher than atmospheric, since a sudden enlargement of valve area would force the diffusive loss of any labile CO_2. Our discussion of the Fick equation has shown, however, that the spiracles alone cannot be responsible for the situation in interburst where 10 or more O_2 molecules may enter the pupa for every CO_2 molecule released. The question may thus arise as to whether, in spite of Punt's observations and of other evidence against gross body movements (*e.g.*, Schneiderman and Williams, 1955), gas transfer might be via ventilatory flow rather than by diffusion. This, however, seems excluded by the fact that the CO_2 burst registers manometrically as an actual increase in gas volume in the respirometer flask, whereas an ordinary exhalation, being brought about by decrease in body volume, would merely exchange gas between tracheae and flask without affecting the over-all volume of the system. The possibility of burst production by some sort of biochemical cataclysm, such as sudden acidification of the blood is, in view of the constant presence of a large reservoir of CO_2 (as shown by the purge experiments), highly improbable. From our original discussion of the Fick equation, therefore, we must conclude that rate of CO_2 release during interburst is determined primarily by diffusion gradients. An analysis of this problem, and of the true role of the spiracles in the cycle, will be considered in a later communication.

Summary

1. Forcing a few of the spiracles of the *Agapema* pupa to remain open abolishes the alternate retention and release ("burst") of CO_2 and greatly augments water loss. The effects are reversed by sealing the inactivated spiracles.

2. Pupae exposed to N_2 after a normal CO_2 burst has been produced release an additional volume of CO_2 twice that of the original burst. The first cycle after such a "purge" is nearly twice as long as normal. These results further implicate the spiracles in CO_2 retention and favor the idea that accumulation of CO_2 triggers the burst.

3. A statistical analysis of successive cycles within individual pupae indicates that burst volume tends to be constant, and comparison of individuals in a population shows significant inverse relations between metabolic rate and cycle length, and possibly between burst volume and cycle length. The significance of these findings is discussed in relation to the triggering of CO_2 release and the rationale of the cycle.

4. Mechanical disturbances may also trigger CO_2 bursts.

5. The effects of temperature and ambient p_{O_2} on interburst CO_2 release rate are interpreted in terms of spiracular response to O_2.

6. The transition from cyclic to continuous CO_2 release is discussed in relation to triggering and to spiracular involvement.

7. The prominence of CO_2 in triggering bursts and of the apparent control of the spiracles by O_2 during interburst; the lack of clear functional relation between interburst release rate, burst volume and cycle length; and the lack of special association between burst volume and either preceding or succeeding cycle length, make it difficult to interpret the burst cycle in terms of simple CO_2 regulation, in which the spiracles act as safety valves to prevent undue accumulation of CO_2. It is suggested that the CO_2 release cycle may be a secondary consequence of minimization of transpiratory water loss.

8. Though the spiracles are intimately involved in CO_2 retention and release it is shown theoretically that regulation of valve area *per se* cannot achieve appreciable CO_2 retention without interfering with O_2 uptake. Further analysis in terms of gas diffusion gradients will be discussed elsewhere.

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